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Screening for androgen agonists using autonomously bioluminescent HEK293 reporter cells

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Abstract

Due to the public health concerns of endocrine-disrupting chemicals, there is an increasing demand to develop improved high-throughput detection assays for enhanced exposure control and risk assessment. A substrate-free, autobioluminescent HEK293$_{ARE/Gal4\text{-}Lux}$ assay was developed to screen compounds for their ability to induce androgen receptor (AR)-mediated transcriptional activation. The assay was validated against a group of 40 recommended chemicals and achieved an overall 87.5% accuracy in qualitatively classifying positive and negative AR agonists. The HEK293$_{ARE/Gal4\text{-}Lux}$ assay was demonstrated as a suitable tool for Tier 1 AR agonist screening. By eliminating exogenous substrate, this assay provided a significant advantage over traditional reporter assays by enabling higher-throughput screening with reduced testing costs while maintaining detection accuracy.

METHOD SUMMARY

A human optimized version of the bacterial luciferase gene cassette was developed such that bioluminescence is controlled by exposure to androgen-disruptor chemicals. This cassette, along with the androgen receptor gene, was co-transfected into an HEK293 human cell host that naturally lacks hormone receptors. The resulting reporter cell line was used to screen compounds for androgenic activity in a low cost, high throughput format.

Keywords

androgen; autobioluminescence; bioreporter; endocrine disrupting chemical (EDC); HEK293; *lux*

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Author contributions

T Xu, G Sayler, S Ripp and D Close conceived the research project and designed the work. T Xu conducted the experiments, analyzed the data and wrote the manuscript. M Gilliam contributed to data analysis and manuscript preparation. S Ripp and D Close made critical contributions to revising the manuscript.

Endocrine-disrupting chemicals (EDCs) are environmental contaminants of significant human health concern. EDCs generally arise from anthropogenic sources such as personal care products, pesticides, pharmaceuticals and industrial manufactured products such as plastics and flame retardants. Upon human exposure, these chemicals may interfere with the function of the endocrine system to alter normal hormone activity, thereby contributing to a wide variety of disorders that affect reproductive, developmental, neurological, cardiovascular, metabolic and immunological health [1,2]. Similar environmental endocrine-disruptive effects have been documented in wildlife and agricultural livestock, as well as in plant development and seed germination [3].

In response to the health-related impacts of EDCs, the US Environmental Protection Agency (EPA) has implemented the Endocrine Disruptor Screening Program for the 21st Century (EDSP21) to identify chemicals that have the potential to interact with the human endocrine system via estrogen, androgen or thyroid bioactivity. Realizing the magnitude of effort and cost in screening thousands of chemicals, EDSP21 leverages *in silico* models and *in vitro* high-throughput assays to more efficiently prioritize and identify chemicals of concern [4–6]. In a previous publication, we reported on a high-throughput amenable *in vitro* assay that used an autobioluminescent human embryonic kidney (HEK293) reporter cell line to screen for chemicals displaying estrogenic endocrine-disruptor bioactivity [7]. This cell line, referred to as $HEK293_{ERE/Gal4\text{-}Lux}$, contained an estrogen response element (ERE)-mediated gene amplification circuit that, upon exposure to estrogenic endocrine-disruptor chemicals (EEDCs), activated a unique synthetic bacterial luciferase operon (*lux*) capable of autonomously generating a bioluminescent signal without the addition of a luciferin substrate and without cell destruction. As such, the $HEK293_{ERE/Gal4\text{-}Lux}$ bioreporter could be added to a microtiter plate along with the library of chemicals one wished to test and then placed in a plate reader instrument for continuous surveillance of estrogenic endocrine-disruptor bioactivity over any time period desired under a fully hands-free assay format. The continuous collection of bioluminescent signaling data from each cell/chemical exposure combination supersedes the informational capacity of existing bioassays that use conventional firefly (*luc*)-based bioluminescent endpoints and/or fluorescent endpoints to create a truly longitudinal, high-throughput assay format that minimizes preparation steps and significantly reduces per assay costs.

In this study, we expand on our EDC screening portfolio by describing the development and validation of an autobioluminescent HEK293 reporter cell line for the identification of chemicals displaying androgenic bioactivity. This bioreporter, referred to as $HEK293_{ARE/Gal4\text{-}Lux}$, contains a synthetic *lux* operon driven by an androgen response element (ARE)-mediated gene amplification circuit. EDCs that disrupt the human androgen signaling pathway typically do so by acting as androgen antagonists, either by binding to and blocking the androgen receptor (AR) or by inhibiting androgens such as testosterone from executing their normal hormonal functions [8]. Chemicals displaying androgen antagonistic activity have, for example, been linked to pulp and paper mill effluents whose entry into water bodies has been implicated in the masculinization of female fish [9]. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) maintains a database of chemicals with known and suspected androgenic agonist potential,

which were used to validate the efficacy and reproducibility of the HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent reporter assay.

Materials & methods

Cell types & culture conditions

HEK293 cells were purchased from the American Type Culture Collection (ATCC, VA, USA) and cultured at 37°C under a 5% CO_2 atmosphere in a humidified incubator. A phenol red-free version of Dulbecco's Modified Eagle's Medium (DMEM/High Modified) (Hyclone, GE Lifesciences, MA, USA) was used as the culture medium. One percent penicillin/streptomycin (Corning, NC USA) and 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS; Atlanta Biologicals, MN, USA) were supplemented for all cultures. Fifty micrograms Zeocin/ml (Thermo Fisher Scientific, MA, USA) and 400 μg G418/ml (Calbiochem, MA USA) were supplemented during clonal selection of cell lines. Twenty-five micrograms Zeocin/ml and 100 μg G418/ml were supplemented for routine maintenance after selection. No antibiotics were supplemented during compound testing. Human adrenocortical carcinoma H295R cells were purchased from ATCC and cultured at 37°C and 5% CO_2 in a humidified incubator. DMEM-F12 medium (Gibco, MA, USA) was used as the culture medium. Nu-Serum I 2.5% (Corning) and 1× ITS+Premix (Corning) were supplemented for all cultures. Before use in steroidogenesis assays and or freezing, cells were maintained for five passages.

Chemicals

Reporter cells were assayed against a library of 40 chemicals recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for the validation of *in vitro* AR agonism assays using mammalian cell reporter gene systems [10]. These chemicals and their sources are listed in Table 1.

Plasmids

Human AR cDNA (transcript variant 1) was synthesized (GenScript, NJ, USA) and cloned into the pcDNA3.1/Zeo vector (Life Technologies, MA, USA) to generate the AR expression vector pcDNA3.1/Zeo/AR. The AR-mediated autobioluminescent reporter plasmid was constructed similarly to the estrogen-receptor-mediated autobioluminescent reporter described previously with the exception that a classical ARE, consisting of 5′-AGAACACTATGTTCTCT-3′, was used in place of the ERE [7]. AR-mediated activation of autobioluminescent production is initiated by a Gal4 amplification circuit to regulate expression of the synthetic *lux* cassette (Figure 1). Three tandem repeats of the ARE and a TATA minimal promoter are located upstream of a Gal4 DNA binding domain that is fused with tandem repeats of the herpes simplex virus VP16 transcription factor motif. A human optimized *lux* cassette [11], regulated by five tandem repeats of the yeast upstream activating sequence (UAS) upstream of a minimal promoter, is contained in a second module.

HEK293$_{ARE/Gal4\text{-}Lux}$ cell line development

HEK293 cells were co-transfected with the AR expression vector pcDNA3.1/Zeo/AR and the AR-mediated autobioluminescent reporter construct ARE-Gal4/UAS-Lux using Viafect

transfection reagent (Promega, WI USA). Stable transfectants were selected with Zeocin (50 μg/ml) and G418 (400 μg/ml). Once individual clones were isolated, the Zeocin and G418 concentrations were reduced to 25 and 100 μg/ml, respectively for routine growth and maintenance.

HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent assay

One hundred microliter volumes of medium containing ~5 × 10^3 HEK293$_{ARE/Gal4\text{-}Lux}$ cells/well were plated in opaque 96-well tissue culture plates (Corning) Following an overnight attachment period, the cells were treated in triplicate with serial dilutions of test chemicals and a vehicle control. For chemical library screening, each individual compound was tested at nine concentrations ranging from 10^{-13} to 10^{-5} or 10^{-12} to 10^{-4} M. Antiandrogen inhibition assays were similarly performed by treating HEK293$_{ARE/Gal4\text{-}Lux}$ cells with 10^{-9} M 5α-dihydrotestosterone or testosterone and the known AR antagonists bicalutamide (10^{-9} to 10^{-3} M) or hydroxyflutamide (10^{-9} to 10^{-5} M). The final solvent concentration was 0.1% in all assays. The cells were incubated with the test compounds for 24 h at 37°C under a 5% CO_2 atmosphere. After exposure, the cells were transferred to an IVIS Lumina imaging system (PerkinElmer, MA USA) for bioluminescent measurement. For assays performed in opaque, 384-well plates (Corning), a similar approach was employed. However, to avoid overcrowding, HEK293$_{ARE/Gal4\text{-}Lux}$ cells were loaded at ~1 × 10^3 cells/well. GraphPad Prism 7 was used to calculate the concentrations required to induce a half maximum effect (EC_{50}) values and the coefficients of variation (CV). $EC_{50(5α\text{-}dihydrotestosterone)}$/$EC_{50(compound)}$ was used to calculate relative effect potency (REP). Z-factor was calculated according to Zhang *et al.* (12).

H295R cell-based steroidogenesis assay

The H295R cell-based steroidogenesis assay was performed as described in Xu *et al.* [7], using norgestrel, hydroxyflutamide and flutamide as test compounds and forskolin and prochloraz as positive and negative controls, respectively.

Results & discussion

Development of the androgen-responsive HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent bioreporter

High-throughput and cost-effective Tier 1 *in vitro* screening assays that can efficiently identify and prioritize chemicals for more in-depth Tier 2 *in vivo* evaluation are valuable tools to meet the EDSP21 program's need for screening thousands of chemicals for potential endocrine-disrupting activities. We previously developed a human cell line (i.e., HEK293)-based estrogen screening assay that produces a continuous bioluminescent signal for detection without the need for adding luciferin exogenously [7]. This was achieved by utilizing a unique synthetic bacterial luciferase reporter operon (*lux*) capable of generating both the luciferase enzyme and the luciferin substrate from endogenous metabolites within the cells [11]. This strategy allowed the reporter signal to be produced autonomously and detected at any time without cell destruction or addition of luciferin substrate. Realizing the potential cost-savings of using autobioluminescent bioreporters to screen thousands of EDCs, in this study, we expanded our EDC screening portfolio to chemicals displaying

androgenic activities because the EPA has not yet identified a standard evaluation procedure for AR transcriptional activation.

Androgen-responsive reporter cells were developed by introducing AR and an ARE-mediated *lux* reporter into naturally AR-negative HEK293 cells. The HEK293 line was selected as the host for this reporter system because its lack of common hormone receptor activities results in low background activity and minimal cross-activation among different hormone signaling pathways and because a variety of luciferase-based AR reporter systems have previously been constructed in this line to allow for facile comparisons with previous reports. However, because the choice of host cell line is known to have an impact on assay performance, it may be necessary to recapitulate our approach in an alternative host, depending on the specific assay constraints encountered [13]. To ensure robust functionality if alternative hosts are used, an AR-induced, Gal4-regulated reporter architecture was used to control *lux* cassette expression. This strategy has previously been used for alternative luciferase- [14,15] and non-luciferase-based [16] androgen agonist reporter systems and consistently shows strong induction in the presence of androgen agonists while maintaining low background expression in their absence. Furthermore, previous work has shown that the Gal4 operator is unresponsive to androgen agonist induction in the absence of AR activation [14], which reduces false-positive identification under high-throughput screening conditions.

Following co-transfection of the AR expression vector and the ARE-Gal4/UAS-Lux reporter construct, several isolates were generated from G418 and Zeocin selection. A total of 18 individual clones were initially screened for their autobioluminescent response. These candidates were treated with 10^{-7} M 17α-methyltestosterone and bioluminescence was measured after 24 h. Four clones displaying the highest fold of induction compared to vehicle control (i.e., 0.1% DMSO treatment) were subjected to a second round of evaluation to compare their sensitivity. The selected clones were treated with nine 17α-methyltestosterone concentrations ranging from 10^{-13} to 10^{-5} M for 24 h to determine the EC_{50} values. The clone displaying the lowest EC_{50} value was designated as $HEK293_{ARE/Gal4\text{-}Lux}$ and used for all further assays.

Performance of the $HEK293_{ARE/Gal4\text{-}Lux}$ androgenic assay against known AR agonists

The dose-response of $HEK293_{ARE/Gal4\text{-}Lux}$ bioreporter cells was first evaluated against a group of known AR agonists, including the natural androgen 5α-dihydrotestosterone, testosterone, 17α-methyltestosterone, 4-androstenedion and mifepristone. The $HEK293_{ARE/Gal4\text{-}Lux}$ bioreporter cells were treated with these test compounds over concentrations from 10^{-13} to 10^{-5} M in the common 96-well plate assay format. All five compounds produced full sigmoidal dose-response curves (Figure 2), which allowed the quantitative calculation of EC_{50} values (Table 2). The natural androgen 5α-dihydrotestosterone was shown to be the most potent AR agonist by the autobioluminescent assay, displaying the lowest EC_{50} value of 7.6×10^{-10} M among the five compounds and ~27-fold of induction at the highest test concentration. The EC_{50} value identified in this study was within the same order of magnitude as the median EC_{50} (1.5×10^{-10} M) from across the 13 mammalian cell reporter gene *in vitro* transcriptional activation assays reported by the ICCVAM meta-analysis [10,17], the EC_{50} (1.38×10^{-10} M) from the fluorescent

reporter/flow cytometry-Hypercyt assay [18], and that of the luciferase-based AR-EcoScreen assay (2.2×10^{-10} M) [19]. Although no reported assays displayed identical EC_{50} values, all successfully identified 5α-dihydrotestosterone as the most potent AR agonist.

With the REP of 5α-dihydrotestosterone defined as 1, the REP values of the other four compounds were calculated as the ratio of the EC_{50} of 5α-dihydrotestosterone to the EC_{50} of the chemical of interest (Table 2). As expected, the natural androgen testosterone was demonstrated as a very potent AR agonist with an EC_{50} of 9.2×10^{-10} M and a REP of 0.826 by the $HEK293_{ARE/Gal4\text{-}Lux}$ assay. This difference is smaller than that observed in some of the alternative systems used in the meta-analysis but is comparable to the ICCVAM-validated median EC_{50} (2×10^{-10} M) and REP (0.75) values (Table 1) [10]. This relatively reduced difference is likely due to the strength of the Gal4-based transcriptional activation step used in our approach. Both the $HEK293_{ARE/Gal4\text{-}Lux}$ assay used in this study and the ICCVAM analyses ranked 17α-methyltestosterone as the third most potent AR agonist after 5α-dihydrotestosterone and testosterone, although the $HEK293_{ARE/Gal4\text{-}Lux}$ assay inferred a higher REP (0.422) than the ICCVAM-validated assays (REP = 0.185). It is worth noting that although 17α-methyltestosterone displayed a lower EC_{50} value than 5α-dihydrotestosterone, induction values at higher concentrations ($\geq 10^{-8}$ M) by 17α-methyltestosterone were greater than those by the most potent agonist 5α-dihydrotestosterone in the $HEK293_{ARE/Gal4\text{-}Lux}$ assay (Figure 2). Similar observations were made in the AR-EcoScreen androgen reporter cells (Chinese hamster ovary [CHO] cells expressing AR and ARE-regulated *luc* reporter), in which medroxyprogesterone acetate and spironolactone exhibited a higher EC_{50} value but showed induction of reporter gene activity greater than the maximal response induced by 5α-dihydrotestosterone [19].

Discrepancies between the $HEK293_{ARE/Gal4\text{-}Lux}$ assay and the ICCVAM guidelines were noted regarding the less potent AR agonists. According to their EC_{50} values, mifepristone was ~three times more potent than 4-androstenedion in this study using the $HEK293_{ARE/Gal4\text{-}Lux}$ bioreporter cells, whereas the ICCVAM guidelines suggested that 4-androstenedion was ~9 times more potent than mifepristone. However, as shown in Figure 2, 4-androstenedion displayed higher fold of induction values than mifepristone at concentrations $\geq 10^{-9}$ M. In fact, the maximal induction by 4-androstenedion and mifepristone were determined to be ~23-fold and approximately sixfold, respectively. It should also be noted that the mifepristone dose-response curve only had an R^2 value of 0.94, whereas the other four compounds displayed sigmoidal curves with R^2 values >0.97, indicating the mifepristone EC_{50} value was less reliable. Our mifepristone dose-response curve was similar to that tested in the AR-EcoScreen cells, with the same characteristics of low induction, narrow dynamic range and decreasing fold of induction at higher concentrations potentially due to cytotoxicity [19]. These results suggest that in these cases, EC_{50} values should not be used as the sole factor for determining chemical potency. Potency evaluation should also include assessment of the goodness of fit of dose-response curves (i.e., R^2 values) and the fold induction.

These considerations are often necessary when comparing findings between different published assays. Many factors can influence EC_{50} value identification among, and even within, assays. For instance, the sensitivity of the detection equipment, quality and purity of

the test compound, environmental factors, technician performance and host cell physiology can vary between runs. When comparing assays, the DNA and amino acid identity of the AR and Gal4 domain regions used, the relative architectures of the operator and reporter open reading frames, and expression and quantum efficiency of the chosen reporter construct can also lead to variability. The ability of the $HEK293_{ARE/Gal4\text{-}Lux}$ assay to produce EC_{50} and REP values within the range of previously reported values while also fully controlling luciferase and luciferin production within the host and providing a continuous luminescent output suggests that these additional capabilities do not significantly perturb host physiology to a level that influences performance.

$HEK293_{ERE/Gal4\text{-}Lux}$ comparison with the H295R steroidogenesis assay

The $HEK293_{ARE/Gal4\text{-}Lux}$ assay was next compared with an ELISA-based steroidogenesis assay that utilizes the H295R human adrenocortical carcinoma cell line. Instead of measuring transcriptional activation via reporter gene activities, the steroidogenesis assay measures a test compound's ability of inducing or inhibiting the capability of H295R cells to produce specific hormones. Both the $HEK293_{ARE/Gal4\text{-}Lux}$ and the H295R cell lines were analogously treated with serial dilutions of norgestrel (strong AR agonist), hydroxyflutamide (very weak AR agonist), and flutamide (negative AR agonistic activity) at concentrations from 10^{-11} to 10^{-5} M to allow for the comparison of results between both assay formats. Norgestrel, which was established as a positive AR agonist using the $HEK293_{ARE/Gal4\text{-}Lux}$ assay, also increased testosterone production in the H295R cells (Figure 3A). For instance, exposure to 10^{-5} M norgestrel induced an ~20-fold increase in autobioluminescent signal in $HEK293_{ARE/Gal4\text{-}Lux}$ cells and an approximately fivefold increase in testosterone production in H295R cells. However, the EC_{50} values of norgestrel in the $HEK293_{ARE/Gal4\text{-}Lux}$ assay and the H295R testosterone assay were estimated to be 2.6×10^{-8} and 7.3×10^{-7} M, respectively. These results indicate that the H295R steroidogenesis assay is less sensitive than the $HEK293_{ARE/Gal4\text{-}Lux}$ transcriptional activation assay in identifying androgenic activity. This hypothesis was further supported by the test results of the very weak AR agonist hydroxyflutamide. Hydroxyflutamide is a potent AR antagonist but has been shown to exhibit low AR transcriptional activation activity at high concentrations (10^{-5} M) in transiently transfected cells [19,20]. In this study, whereas exposure to 10^{-5} M hydroxyflutamide did not change testosterone levels in H295R cells, the same hydroxyflutamide concentration induced an approximately twofold increase in autobioluminescent signal in the $HEK293_{ARE/Gal4\text{-}Lux}$ bioreporter cells (Figure 3B). The H295R steroidogenesis assay did not demonstrate a detectable change in testosterone production in response to flutamide treatment, which also failed to induce autobioluminescent production in the $HEK293_{ARE/Gal4\text{-}Lux}$ assay (Figure 3C).

The H295R steroidogenesis assay was chosen for comparison because it is an EPA-approved method for evaluating androgen agonist activity [21]. In addition to the improved sensitivity of the $HEK293_{ARE/Gal4\text{-}Lux}$ assay over the H295R steroidogenesis assay, this comparison also suggested improved suitability for high-throughput performance to meet the Tier 1 EDC screening demand. For example, the $HEK293_{ARE/Gal4\text{-}Lux}$ assay did not require any sample preparation time beyond plating and dosing the cells, whereas the H295R steroidogenesis assay required at least 3 h of hands-on time of sample preparation before signal detection.

The autobioluminescent HEK293$_{ARE/Gal4\text{-}Lux}$ assay also substantially reduced the assay cost by eliminating any additional reagent for signal detection. In contrast, the H295R steroidogenesis assay required the recurring cost of ELISA kits at $500 per kit for assaying one 96-well plate with four chemical treatments.

Performance testing of the HEK293$_{ARE/Gal4\text{-}Lux}$ androgenic assay against a chemical library

To succeed as a Tier 1 screening assay, HEK293$_{ARE/Gal4\text{-}Lux}$ bioreporter cells must be capable of rapidly and efficiently reporting the androgenic agonist potential of a wide range of chemicals. The ICCVAM recommends a list of 78 chemicals for use in validation of mammalian cell reporter gene system-based *in vitro* AR transcriptional activation agonism assays [10]. Of the 78 compounds, quantitative guidelines (EC_{50} values) are available for only six compounds, whereas 39 compounds on this list have only qualitative (i.e., positive or negative response) data, and the remaining 33 chemicals have only anticipated responses without any qualitative data available. To assess the suitability of the HEK293$_{ARE/Gal4\text{-}Lux}$ assay as a Tier I screening tool, in this study 40 compounds representative of the ICCVAM recommended list were tested, including five of the six compounds with EC_{50} guidelines, 24 of the 39 compounds with qualitative guidelines, and 11 of the 33 compounds with only presumptive classifications. Overall, the test compound library included 16 positive (14 reported and two anticipated) and 24 negative (15 reported and nine anticipated) AR agonists from the ICCVAM recommended list.

Across the full list, 35 of the 40 (87.5%) tested compounds were qualitatively identified by the autobioluminescent HEK293$_{ARE/Gal4\text{-}Lux}$ assay as positive or negative for AR agonist activity in agreement with the ICCVAM meta-analysis (Table 3). Specifically, 14 of the 16 (87.5%) reported and presumptive positive agonists and 21 of the 24 (87.5%) reported and presumptive negative compounds were identified correctly. A total of five compounds generated responses contradicting the reported or predicted ICCVAM classification.

Of the 14 ICCVAM-reported positive AR agonists including five chemicals with EC_{50} values and nine compounds with only qualitative classification, the HEK293$_{ARE/Gal4\text{-}Lux}$ assay successfully classified 13 compounds with positive AR agonism. As described earlier, all five quantitatively determined AR agonists generated full sigmoidal dose-response curves (Figure 2), allowing estimation of their EC_{50} values (Table 2). Additionally, the HEK293$_{ARE/Gal4\text{-}Lux}$ assay yielded correct classifications for eight of the nine compounds with qualitative data (Table 3), including hydroxyflutamide (Figure 3B); hormones 17β-estradiol and progesterone (Figure 4A); and pharmaceuticals fluoxymesterone, dexamethasone, cyproterone acetate, spironolactone and nilutamide (Figures 4B and C).

Crosstalk between sex-hormone-regulated signaling pathways are not uncommon [22]. Estrogens are known to interact with other hormone receptors in addition to estrogen receptors, such as progesterone receptors (PR), glucocorticoid receptors (GR) and AR. In this study, the natural estrogen 17β-estradiol activated AR-mediated transcriptional response at concentrations $\geq 10^{-8}$ M and reached a maximal induction of approximately ninefold at the highest test concentration of 10^{-5} M (Figure 4A). Progesterone, an important hormone involved in menstrual cycle and pregnancy, was shown to be a less potent agonist than 17β-estradiol. Detectable induction was only observed at concentrations $\geq 10^{-6}$ M, whereas

a maximal induction of approximately sevenfold was generated by exposure to 10^{-5} M progesterone. The induction decreased to approximately fourfold at 10^{-4} M, potentially due to toxicity.

Several pharmaceuticals targeting the hormone receptors also exhibited AR agonism as expected. As shown in Figure 4B, fluoxymesterone, a synthetic androgen and a steroid medication, exhibited the highest increase in autobioluminescent reporter signal of ~23-fold at 10^{-5} M, similar to the maximal response induced by 5α-dihydrotestosterone. Because of the lack of signal plateau, the EC_{50} value could not be reliably calculated for fluoxymesterone. However, the dose-response curve indicated that 10^{-7} to 10^{-6} M fluoxymesterone induced 50% of the maximal 5α-dihydrotestosterone response, which had an EC_{50} of 7.6×10^{-10} M. These data suggest that despite the strong reporter signal induction, fluoxymesterone was a less potent AR agonist. Similar observations were also found in the AR-EcoScreen assay, which showed similar maximal induction of fluoxymesterone and a higher EC_{50} value (i.e., less potent) compared with 5α-dihydrotestosterone [19]. Other pharmaceuticals including cyproterone acetate, dexamethasone, spironolactone and nilutamide exhibited weak agonist activities only at higher concentrations and did not reach 50% of maximal 5α-dihydrotestosterone induction (Figure 4C).

Two ICCVAM-predicted positive AR agonists, 17β-trenbolone and ketoconazole, were also tested in this study. In agreement with the ICCVAM prediction, 17β-trenbolone, which is a steroid with strong AR binding affinity, induced autobioluminescent signal exceeding the maximal response of 5α-dihydrotestosterone in the $HEK293_{ARE/Gal4\text{-}Lux}$ assay (Figure 4D). Significantly increased autobioluminescent signal over vehicle controls was observed at concentrations beginning at 3.7×10^{-11} M, with reporter activity continuing to increase up to the highest test concentration (3.7×10^{-6} M) without leveling off. As a result, an EC_{50} value was not calculated for this compound due to the absence of a typical sigmoidal dose-response curve. In contrast to ICCVAM predictions, ketoconazole did not induce autobioluminescent signal in the $HEK293_{ARE/Gal4\text{-}Lux}$ bioreporter cells. According to the ICCVAM guideline, ketoconazole is presumed to be a positive AR agonist based on positive AR agonism response in yeast assays [10,17]. However, we were unable to identify any study in the literature showing AR agonistic activity of ketoconazole in mammalian-cell-based transcriptional activation assays. In fact, the CHO-cell-based AR-EcoScreen assay also classified ketoconazole as a negative AR agonist [19].

The $HEK293_{ARE/Gal4\text{-}Lux}$ assay achieved an overall 87.5% accuracy in classifying negative AR agonists, correctly identifying 12 of 15 and nine of nine reported and presumptive negative AR agonists in the ICCVAM meta-analysis, respectively. Exceptions included 17α-ethinylestradiol, butyl benzyl phthalate and corticosterone (Figure 5). 17α-ethinylestradiol and butyl benzyl phthalate only induced a detectable increase in bioluminescent signal at the highest test concentration of 10^{-5} M in this study. Interestingly, the AR-EcoScreen assay identified 17α-ethinylestradiol as an AR antagonist, suggesting its ability to bind to AR [19]. Therefore, the positive AR agonist classification in the $HEK293_{ARE/Gal4\text{-}Lux}$ assay could possibly be due to the recruitment of differential transcriptional cofactors and coregulators. Corticosterone displayed positive agonistic activity at 10^{-7} M and higher

concentrations but did not reach 50% of maximal 5α-dihydrotestosterone response. As a known GR agonist, the positive AR agonistic activity of corticosterone is not surprising because crosstalk between GR and AR signaling pathways has been well documented [22]. Positive AR transcriptional activity of corticosterone has also been demonstrated in other mammalian-cell-based assays, such as the CHO-cell-based AR-EcoScreen assay [19] and the MDA-lMB-453 breast-cancer-cell-based MDA-kb2 assay [23], with both cell types expressing GR endogenously. In contrast, corticosterone failed to elicit positive AR agonistic effects in several yeast-based reporter assays [24–26], possibly due to a lack of endogenous GR expression in the *Saccharomyces cerevisiae* host cells.

Performance of the HEK293$_{ARE/Gal4\text{-}Lux}$ assay against known AR antagonists

In addition to profiling AR agonists, the utility of the HEK293$_{ARE/Gal4\text{-}Lux}$ reporter cells to identify AR antagonists was also evaluated. The autobioluminescent signal induced by 5α-dihydrotestosterone was reduced by the antiandrogens bicalutamide and hydroxyflutamide in a dose-responsive manner, with IC_{50} values of 3.8×10^{-7} and 5.5×10^{-8} M, respectively (Figure 6A). Similarly, the IC_{50} values of bicalutamide and hydroxyflutamide against testosterone were determined to be 2.6×10^{-7} and 2.6×10^{-8} M, respectively (Figure 6B). The antiandrogenic potency of hydroxyflutamide determined in this study was comparable to that reported for the AR-EcoScreen assay, which identified an IC_{50} of 4.9×10^{-8} M using 5α-dihydrotestosterone as the reference androgen [19]. The IC_{50} hydroxyflutamide from this study was well within the 1×10^{-8} to 4.5×10^{-5} M range reported across all mammalian cell reporter gene transcriptional antagonism studies in the ICCVAM meta-analysis [10]. Studies cited by the ICCVAM guideline listed bicalutamide IC_{50} values ranging from 5×10^{-7} to 1.8×10^{-5} M, whereas this study yielded a slightly lower IC_{50} value. The use of different reference androgens and the differences in physiology between the reporter host cells used among studies likely contributed to this discrepancy. However, both our study and the ICCVAM meta-analysis successfully identified bicalutamide as a less potent antiandrogen than hydroxyflutamide. These results demonstrate that the HEK293$_{ARE/Gal4\text{-}Lux}$ assay is suitable for profiling AR antagonists.

Intraassay & interassay variability of the HEK293$_{ARE/Gal4\text{-}Lux}$ assay

The intraassay and interassay variability of the HEK293$_{ARE/Gal4\text{-}Lux}$ assay was also evaluated. Multiple replicate 96-well plate assays were performed against the known strong inducer chemical 17α-methyltestosterone from 10^{-13} to 10^{-5} M using individual batches of HEK293$_{ARE/Gal4\text{-}Lux}$ bioreporter cells. Each assay was performed under identical conditions, and the results were compared. The average intraassay coefficient of variation (CV) was determined to be 19.9 (± 1.1)% (n = 63) and interassay CV was determined to be 26.7% (n = 63). The Z-factor of the assay was calculated to be 0.80 (± 0.03), which was within the ideal range of >0.5 [12]. To further evaluate assay performance under higher throughput metrics, multiple 384-well plate assays were performed against 17α-methyltestosterone. The intraassay and interassay CVs were determined to be 24.3 (± 1.6)% and 20.7% (n = 63), respectively. Compared with the 96-well plate format, the 384-well plate assay resulted in a reduced Z-factor of 0.47 (± 0.16), which could be because fewer cells per well were used for the assay.

Conclusion

Because of the public health concerns of EDCs and their ubiquitous presence in household products, personal care products and the environment, there is an increasing demand to develop improved high-throughput detection assays for enhanced exposure control and risk assessment. In this study, a substrate-free, autobioluminescent HEK293$_{ARE/Gal4\text{-}Lux}$ assay was developed and characterized as a method to screen compounds for their capability to induce AR-mediated transcriptional activation. The assay was validated against a group of 40 chemicals recommended by the ICCVAM guidelines and achieved an overall 87.5% accuracy in qualitatively classifying positive and negative AR agonists. The HEK293$_{ARE/Gal4\text{-}Lux}$ assay, by eliminating exogenous substrate addition, provided a significant advantage over traditional reporter assays by enabling higher throughput screening with reduced testing costs while maintaining the accuracy of AR agonist detection. Traditional EDC screening assays are often limited by the types of cells used, especially with regard to variable endogenous hormone receptor expression in different tissue types. With traditional assays that require exogenous luciferin substrate to generate reporter signal, the cost of screening the same chemical in multiple tissue types is not insignificant. On the contrary, the HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent reporter system can be expanded into additional cell types for tissue-specific EDC screening without incurring additional substrate costs. The primary weakness of the HEK293$_{ARE/Gal4\text{-}Lux}$ assay is its variability, which was likely caused by using multiple cell batches and different personnel preparing the assays. Standardizing cell-counting methods and automating the liquid handling would further improve variability. Overall, it was demonstrated that the autobioluminescent HEK293$_{ARE/Gal4\text{-}Lux}$ assay was a suitable tool for Tier 1 AR agonist screening. Future studies will focus on validating the HEK293$_{ARE/Gal4\text{-}Lux}$ assay for AR antagonist detection and improving assay variability.

Financial & competing interests disclosure

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Future perspective

Over the last few decades, human-generated EDCs have become prevalent within the environment. Looking forward, anthropogenic EDC deposition from agricultural, pharmaceutical and industrial sources is likely to continue at an alarming pace. Although the $HEK293_{ARE/Gal4\text{-}Lux}$ assay developed in this work provides a higher throughput method for identifying these compounds, it cannot remove them from the environment or prevent their utilization. Having recognized the need to regulate these compounds, their identification is only the first step toward reducing their use and environmental deposition. Ultimately, methods for remediation and transitions to alternative chemicals without these physiological downsides will be necessary to prevent the negative health impacts they pose. Significant research will be required in this area to identify functional alternatives with lower negative impacts.

Summary points

- An autonomously bioluminescent HEK293 reporter cell line, $HEK293_{ARE/Gal4\text{-}Lux}$, was created to identify chemicals displaying androgenic bioactivity by self-initiating a bioluminescent signal.
- The new reporter line yielded consistent results compared with other published reporter systems.
- The ability to autonomously activate bioluminescent signals in the presence of chemicals displaying androgenic bioactivity simplified assay design relative to alternative approaches.
- Removing the need for external activation reduced performance costs by eliminating the need for luciferin addition and sample destruction concurrent with testing.
- The intraassay and interassay performance metrics of the system fell within acceptable parameters.

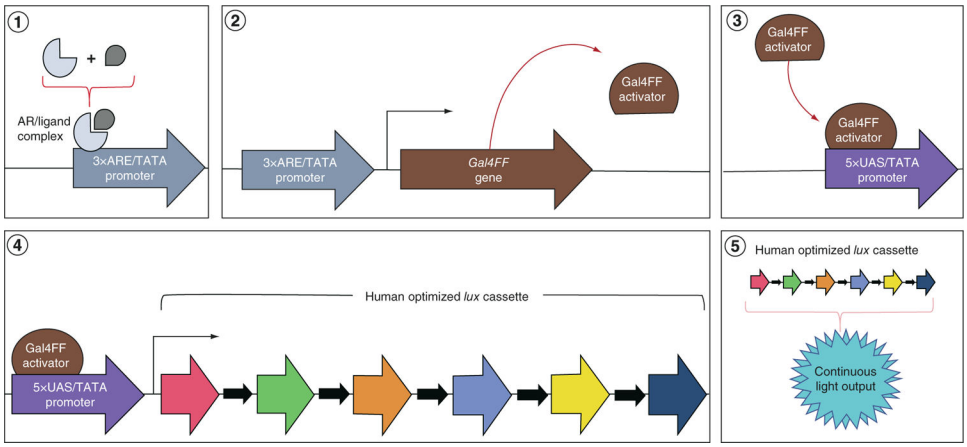

Figure 1. Schematic representation of the autobioluminescent androgen bioreporter construct.

In this design, the androgen receptor (AR) is activated by androgen or androgenic compounds to form an AR/ligand complex that then activates the 3×ARE/TATA promoter **(1)**. The 3×ARE/TATA promoter subsequently initiates the transcription of the *Gal4FF* gene, which is a transcriptional activator **(2)**. The Gal4FF activator then binds and activates the 5×UAS/TATA promoter **(3)**, which ultimately initiates transcription of the human optimized *lux* cassette **(4)** to generate continuous light output **(5)**.

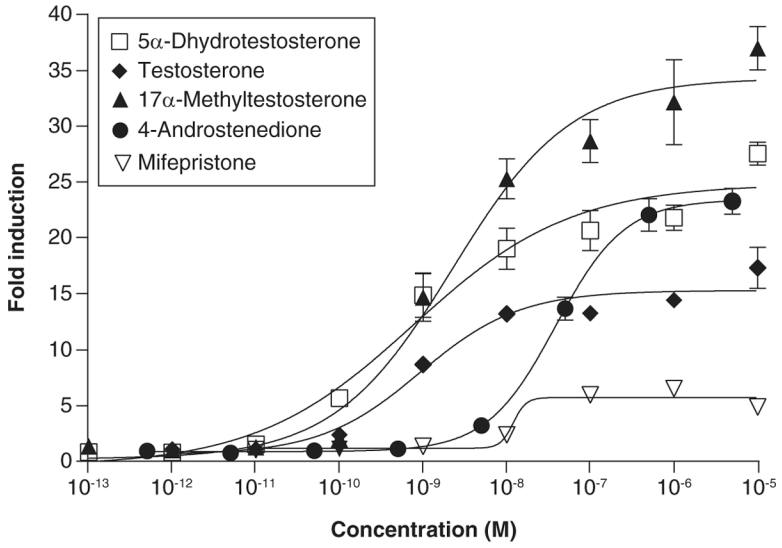

Figure 2. Dose-response curves of five known androgen receptor (AR) agonists against the HEK293$_{ARE/Gal4\text{-}Lux}$ bioreporter cells.

5α-Dihydrotestosterone was identified as the most potent AR agonist, displaying an EC_{50} similar to the Interagency Coordinating Committee on the Validation of Alternative Methods meta-analysis median value. Testosterone demonstrated an EC_{50} of 9.2×10^{-10} M and a relative effect potency (REP) of 0.826. 17α-Methyltestosterone was the third most potent AR agonist, with a REP of 0.422 (n = 3, mean ± standard deviation). Curves were fitted using the four-parameter sigmoidal regression.

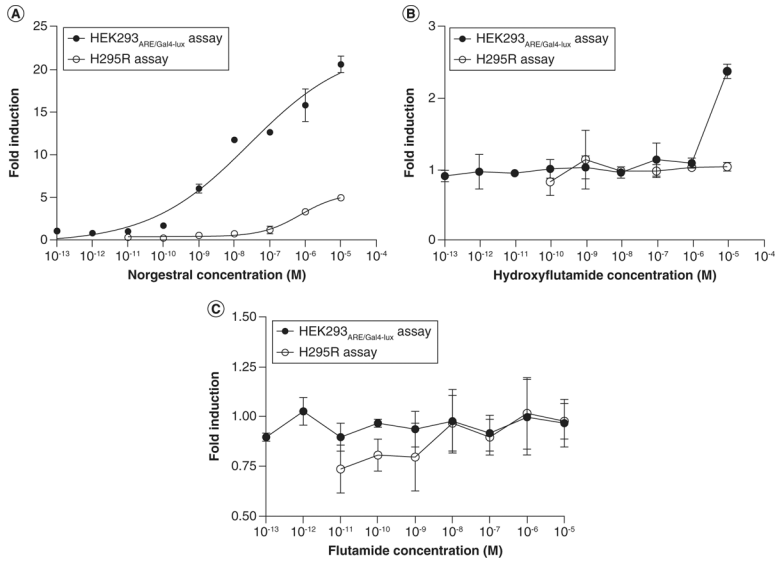

Figure 3. HEK293$_{ARE/Gal4-Lux}$ and H295R assay comparison.

Comparison of the HEK293$_{ARE/Gal4-Lux}$ autobioluminescent assay and the H295R steroidogenesis assay for norgestrel **(A)**, hydroxyflutamide **(B)** and flutamide **(C)**. Norgestrel was identified as an androgen receptor (AR) agonist and testosterone stimulant. Hydroxyflutamide was a potent AR antagonist but did not alter testosterone production. Flutamide did not induce autobioluminescence or change testosterone production (n = 3, mean ± standard deviation).

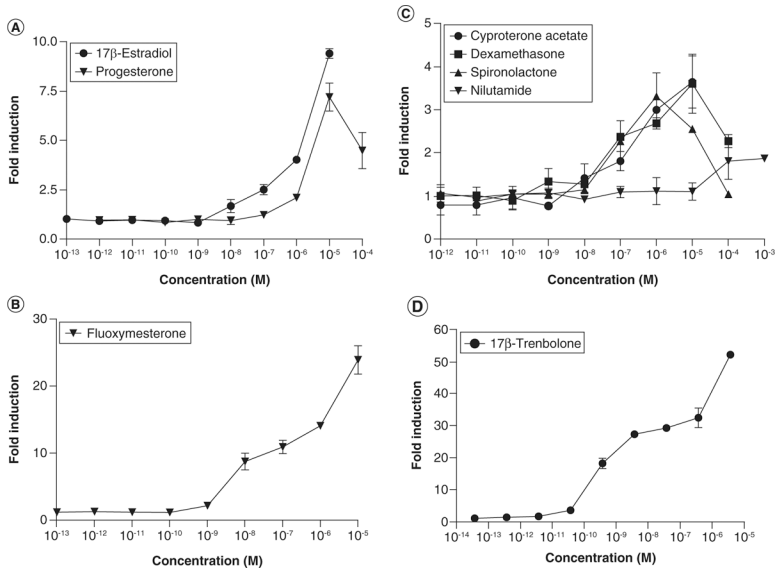

Figure 4. HEK293$_{ARE/Gal4\text{-}Lux}$ assay performance against qualitatively identified androgen receptor agonists.

Response curves of qualitatively identified androgen receptor (AR) agonists **(A–C)** and an Interagency Coordinating Committee on the Validation of Alternative Methods-predicted AR agonist **(D)** using the HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent assay. 17β-Estradiol activated AR-mediated transcriptional response at $\geq 10^{-8}$ M, whereas progesterone induction was only observed at $\geq 10^{-6}$ M. Fluoxymesterone also exhibited a significant increase in autobioluminescent reporter signal. The pharmaceutical compounds cyproterone acetate, dexamethasone, spironolactone and nilutamide all exhibited weak agonist activities at high concentrations, whereas 17β-Trenbolone strongly induced autobioluminescence (n = 3, mean ± standard deviation).

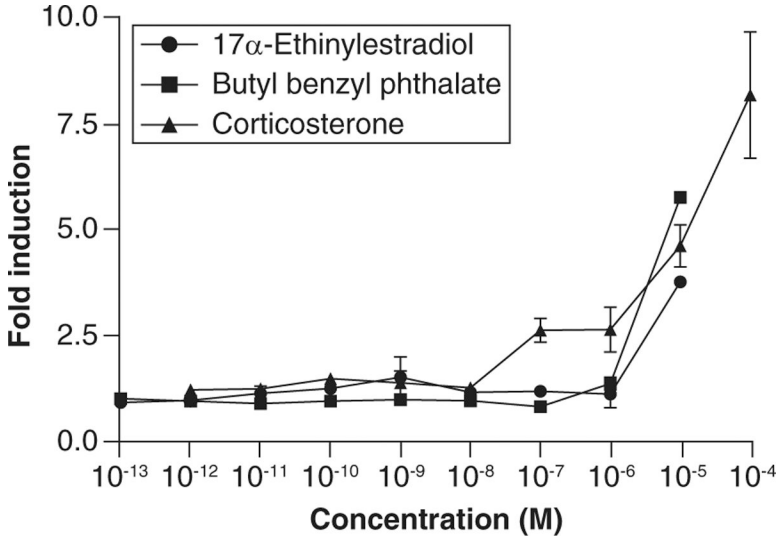

Figure 5. Response of 17α-ethinylestradiol, butyl benzyl phthalate and corticosterone against the HEK293$_{ARE/Gal4\text{-}Lux}$ bioreporter cells that produced contradictory results against the Interagency Coordinating Committee on the Validation of Alternative Methods guidelines.

17α-Ethinylestradiol and butyl benzyl phthalate only induced a detectable increase in bioluminescent signal at the highest test concentration of 10^{-5} M. Corticosterone displayed positive agonistic activity at $\geq 10^{-7}$ M (n = 3, mean ± standard deviation).

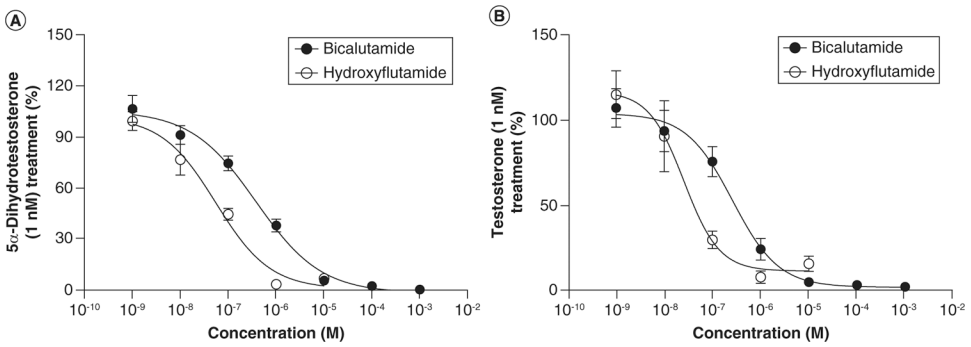

Figure 6. HEK293$_{\text{ARE/Gal4-Lux}}$ assay peformance against androgen receptor antagonists.

Antiandrogenic response curves of bicalutamide and hydroxyflutamide against 5α-Dihydrotestosterone **(A)** and testosterone **(B)** using the HEK293$_{\text{ARE/Gal4-Lux}}$ autobioluminescent assay. Autobioluminescence induced by 5α-Dihydrotestosterone was reduced by bicalutamide and hydroxyflutamide in a dose-responsive manner. The IC_{50} values of bicalutamide and hydroxyflutamide against testosterone were 2.6×10^{-7} and 2.6×10^{-8} M, respectively (n = 3, mean ± standard deviation).

Table 1.

List of chemicals used in this study.

Chemical	CAS no.	Product Class (ICCVAM 2003)	Source†	Catalog no.
17ß-Estradiol	50-28-2	Hormone	Sigma	E8875
17ß-Trenbolone	10161-33-8	Pharmaceutical	Sigma	T-043
17α-Estradiol	57-91-0	Hormone	Sigma	E8750
17α-Ethynylestradiol	57-63-6	Pharmaceutical	Sigma	E4876
2,4,5-Trichloro-phenoxyacetic acid	93-76-5	Pesticide	SCBT	SC-209335
4-Androstenedione	63-05-8	Hormone	Sigma	A-075
4-Cumylphenol	599-64-4	Chemical intermediate	SCBT	SC-232618
4-Hydroxytamoxifen	68047-06-3	Pharmaceutical	SCBT	SC-3542
p-n-Nonylphenol	104-40-5	Chemical intermediate	SCBT	SC-256816
5α-Dihydrotestosterone	521-18-6	Pharmaceutical	Sigma	D-073
Actinomycin D	50-76-0	Pharmaceutical	SCBT	SC-200906
Apigenin	520-36-5	Natural product	SCBT	SC-3529
Benzyl butyl phthalate	85-68-7	Plasticizer	TargetMol	T0640
Bicalutamide	90357-06-5	Pharmaceutical	SCBT	SC-202976
Bisphenol A	80-05-7	Chemical intermediate	SCBT	SC-391751
Kepone (Chlordecone)	143-50-0	Pesticide	SCBT	SC-394278
Clomiphene citrate	50-41-9	Pharmaceutical	SCBT	SC-205636
Corticosterone	50-22-6	Pharmaceutical	SCBT	SC-300391
Coumestrol	479-13-0	Natural product	SCBT	SC-205638
Cyproterone acetate	427-51-0	Pharmaceutical	SCBT	SC-204703
Daidzein	486-66-8	Natural product	SCBT	SC-24001
Dexamethasone	50-02-2	Pharmaceutical	SCBT	SC-29059
Diethylstilbestrol	56-53-1	Pharmaceutical	SCBT	SC-204720
Finasteride	98319-26-7	Pharmaceutical	SCBT	SC-203954
Fluoxymestrone	76-43-7	Pharmaceutical	TargetMol	T2581
Flutamide	13311-84-7	Pharmaceutical	SCBT	SC-204757
Genistein	446-72-0	Natural product	SCBT	SC-3515
Haloperidol	52-86-8	Pharmaceutical	Sigma	H-030
meso-Hexestrol	84-16-2	Pharmaceutical	SCBT	SC-205713
Hydroxyflutamide	52806-53-8	Pharmaceutical; metabolite	SCBT	SC-205574
Kaempferol	520-18-3	Natural product	SCBT	SC-202679
17α-Methyltestosterone	58-18-4	Pharmaceutical	Sigma	M7252
Mifepristone	84371-65-3	Pharmaceutical	SCBT	SC-203134
Nilutamide	63612-50-0	Pharmaceutical	SCBT	SC-203664
o,p′-DDT	789-02-6	Pesticide	SCBT	SC-257937
Procymidone	32809-16-8	Pesticide	SCBT	SC-236465

Chemical	CAS no.	Product Class (ICCVAM 2003)	Source†	Catalog no.
Progesterone	57-83-0	Pharmaceutical	SCBT	SC-296138
Spironolactone	52-01-7	Pharmaceutical	SCBT	SC-204294
Testosterone	58-22-0	Pharmaceutical	Sigma	T1500
Vinclozolin	50471-44-8	Pesticide	SCBT	SC-251425

†Chemicals sourced from SCBT (Santa Cruz Biotechnology, CA USA), Sigma (Millipore-Sigma, MA USA), and TargetMol (Boston, MA USA).

ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods.

Table 2.

Performance of the HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent assay against known AR agonists.

Chemical	HEK293$_{ARE/Gal4\text{-}Lux}$ EC_{50} (M)[†]	HEK293$_{ARE/Gal4\text{-}Lux}$ REP[†]	ICCVAM median EC_{50} (M)[‡]	ICCVAM REP (based on median EC_{50} values)[‡]	AR-EcoScreen assay EC_{50} (M)[§]
5α-Dihydrotestosterone	7.6×10^{-10}	1	1.5×10^{-10}	1	2.2×10^{-10}
Testosterone	9.2×10^{-10}	0.826	2×10^{-10}	0.750	1.1×10^{-9}
17α-Methyltestosterone	2.2×10^{-9}	0.422	8.1×10^{-10}	0.185	7.1×10^{-10}
4-Androstenedion	3.9×10^{-8}	0.020	1.5×10^{-9}	0.100	1.0×10^{-9}
Mifepristone	1.2×10^{-8}	0.063	1.4×10^{-8}	0.011	3.2×10^{-5}

[†]Data derived from this study.

[‡]Data derived from Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) guidelines [10].

[§]Data derived from Dennis *et al.* [18].

REP: Relative effect potency.

Table 3.

Qualitative androgen receptor (AR) agonism responses of the HEK293$_{ARE/Gal4\text{-}Lux}$ autobioluminescent assay relative to the ICCVAM meta-analysis for all tested compounds.

Chemical	**Meta-analysis majority response from ICCVAM**[†]	**HEK293$_{ARE/Gal4\text{-}Lux}$ response from this study**[‡]
Compounds with quantitative data (5)		
5α-Dihydrotestosterone	Pos.	Pos.
Testosterone	Pos.	Pos.
17α-Methyltestosterone	Pos.	Pos.
4-Androstenedione	Pos.	Pos.
Mifepristone	Pos.	Pos.
Compounds with only qualitative data (24)		
17β-Estradiol	Pos.	Pos.
Cyproterone acetate	Pos.	Pos.
Progesterone	Pos.	Pos.
Hydroxyflutamide	Pos.	Pos.
Dexamethasone	Pos.	Pos.
Spironolactone	Pos.	Pos.
Fluoxymestrone	Pos.	Pos.
Nilutamide	Pos.	Pos.
Flutamide	Neg.	Neg.
Diethylstilbestrol	Neg.	Neg.
Kepone (Chlordecone)	Neg.	Neg.
Bisphenol A	Neg.	Neg.
Corticosterone [§]	**Neg.**	**Pos. ≥100 nM**
o,p'-DDT	Neg.	Neg.
17α-Ethinylestradiol [§]	**Neg.**	**Pos. at 10 μM**
4-Hydroxytamoxifen	Neg.	Neg.
p-n-Nonylphenol	Neg.	Neg.
Procymidone	Neg.	Neg.
Vinclozolin	Neg.	Neg.
Butyl benzyl phthalate [§]	**Neg.**	**Pos. at 10 μM**
Coumestrol	Neg.	Neg.
4-Cumylphenol	Neg.	Neg.
17α-Estradiol	Neg.	Neg.
Compounds with anticipated response only (11)		
Ketoconazole [§]	**Pos. (anticipated)**	**Neg.**
17β-Trenbolone	Pos. (anticipated)	Pos.

Chemical	**Meta-analysis majority response from ICCVAM**[†]	**HEK293$_{ARE/Gal4\text{-}Lux}$ response from this study**[‡]
Actinomycin D	Neg. (anticipated)	Neg.
Finasteride	Neg. (anticipated)	Neg.
2,4,5-Trichlorophenoxy-acetic acid	Neg. (anticipated)	Neg.
Apigenin	Neg. (anticipated)	Neg.
Clomiphene citrate	Neg. (anticipated)	Neg.
Daidzein	Neg. (anticipated)	Neg.
Genistein	Neg. (anticipated)	Neg.
meso-Hexestrol	Neg. (anticipated)	Neg.
Kaempferol	Neg. (anticipated)	Neg.

[†]Data derived from AR agonism meta-analyses from the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [10].

[‡]Data derived from this study.

[§]Chemicals displaying contradictory response profiles.